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Mercury Derivatives of the Fab and Fc Fragments of a Human Myeloma Protein*

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ABSTRACT: To explore the role of the covalent interchain bonding in the structural organization of immunoglobulins, and to develop a general method for attaching heavy metals to these proteins, the reactions of the interchain disulfide bridges with mercuric ion have been investigated. In the Fab and Fc fragments of a human myeloma protein of subclass IgG1, a single atom of mercury forms a stable complex with the pair of cysteine residues derived from each interchain bridge. The mercury derivative of Fab resembles native Fab except that the disulfide bond joining light chain to Fd is replaced by a bridge of the type S–Hg–S. In contrast, the introduction of mercury into Fc induces a rearrangement of the disulfide bonding. When the two closely spaced interchain bridges in this fragment are reacted with Hg^{2+}, they re-form as intrachain bridges with mercury held in a compact ring between two cysteine and two proline residues. The Fc fragment is accordingly converted into a pair of monomeric subunits. Provided that they are not exposed to dissociating solvents, the mercury derivatives are indistinguishable from the native proteins by a number of structural and immunochemical criteria. The crystal structure of the derivative of Fc was closely related to, but not isomorphous with, that of native Fc.

Immunoglobulins are composed of one or more units of a basic tetrameric molecule consisting of two heavy and two light polypeptide chains (reviewed by Edelman and Gall, 1969).[1] Although the heavy and light chains are generally cross-linked by disulfide bonds, noncovalent interactions among the chains are usually sufficient to preserve the tetrameric structure and the activity of these proteins. Indeed, cleavage of the interchain disulfide bonds of antibodies by reduction and alkylation may have little or no effect on antigen binding (Weir and Porter, 1966; Jaton *et al.*, 1968). Moreover, in certain classes of immunoglobulin, some of the light and heavy chains are not linked to each other by disulfide bridges, but are held together by noncovalent interactions alone (Abel and Grey, 1968; Grey *et al.*, 1968). However, some functions of antibodies, such as complement fixation, may be weakened by disruption of the interchain bonding (Schur and Christian, 1964).

To obtain further information on the role of the interchain bridges in the structural organization of these molecules, we have initiated studies in which these bonds are modified by the introduction of a bifunctional reagent. A simple reagent of this type with a high degree of specificity for the sulfhydryl group is the divalent mercuric ion. Several proteins have been modified by reduction and reaction with Hg^{2+} to form derivatives with one or more disulfide bonds replaced by bridges

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[1] The nomenclature used is that recommended by the World Health Organization (1964, 1965, 1966).

of the type S–Hg–S (Arnon and Shapira, 1969; Sperling *et al.*, 1969; Sperling and Steinberg, 1971). The addition of mercury increases the length of the bond by about 3 Å (Yakel and Hughes, 1954; Bradley and Kunchur, 1965), and the effects on the protein have varied according to the location and number of the modified residues. In some cases the addition of mercury has been accompanied by little or no change in the gross structural features or biological properties of the proteins (Arnon and Shapira, 1969; Sperling *et al.*, 1969).

An additional objective of the present studies is to develop a generally applicable method for preparing heavy metal derivatives for X-ray diffraction studies of immunoglobulins or other proteins. The addition of mercury to specific disulfide bridges offers a practical rational alternative to the empirical methods that are frequently used to prepare these derivatives (Blake, 1968). Moreover, the possibility of attaching the heavy metal atom to one or more defined residue positions should be particularly useful for crystallographic studies.

We report here that mercuric ion may be added specifically to the interchain disulfide bonds of the Fab and Fc fragments of a human myeloma protein of subclass IgG1. A single atom of mercury forms a stable complex with the pair of cysteine residues derived from each interchain bridge. The properties of the resulting derivatives vary according to the number and position of the interchain linkages.

Experimental Section

Preparation of Gil Myeloma Protein. Plasma from a patient with multiple myeloma was provided by Dr. W. Moloney and Dr. C. Alper of Harvard Medical School. Bovine thrombin (Parke Davis, Detroit, thrombin topical), 716 units, was added to 422 ml of plasma, and the resulting clot was removed. Serum (99 ml) was passed through a column containing 116 g of DEAE-cellulose (Whatman DE 52, Reeve Angel, Clifton, N. J.) equilibrated with 0.02 M Tris-chloride (pH 8.0). The purified myeloma protein was eluted from the column with the same buffer in a yield of 37 mg/ml of serum.

Preparation of Fab and Fc Fragments. The fragments were prepared by digestion with papain essentially as described for another IgG1 myeloma protein (Press *et al.*, 1966) except that the weight ratio of enzyme to immunoglobulin was 1:200 and the time of digestion was 10 min. The papain digest was dialyzed against 0.02 M Tris-chloride (pH 8.0) and applied to a column of DEAE-cellulose equilibrated in the same buffer. (For 100 mg of protein digest, 4 g of exchange resin was used.) Of the total absorbancy units applied to the column, 66% were eluted with the starting buffer and this material consisted mainly of the Fab fragment (see Results). A gradient of NaCl was then applied and the remaining 34% of the absorbance units, later identified as Fc, emerged as a single peak at a salt concentration of 0.075 M. The fraction that emerged in the void volume contained a small amount of undigested IgG; this was removed by gel filtration with Sephadex G-100 in 0.02 M Tris-chloride (pH 8.0).

Partial Reduction and Alkylation. A 1–2% solution of protein (Gil IgG, Fab or Fc) in 0.1 M Tris-chloride (pH 8.0) was made 5 mM in DTT[2] (Calbiochem, A grade, Los Angeles, Calif.) and incubated for 60 min at 37°. The concentration of DTT was checked by measurement of the optical density at 412 nm after reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Calbiochem, A grade) as described by Ellman (1959). Reduction was terminated by the addition of iodoacetamide (Aldrich Chemical Co., Milwaukee, Wis., recrystallized from water) or iodoacetic acid (Eastman Organic Chemicals, recrystallized from ether–petroleum ether, bp 37–47) to a final concentration of 12–15 mM and incubation for 30–60 min at room temperature. Sometimes [1-^{14}C]iodoacetic acid or [1-^{14}C]iodoacetamide was used. The protein was then dialyzed against 0.1 M Tris-chloride, pH 8.0. The introduction of Hg^{2+} into partially reduced Fab and Fc is described under Results.

Preparation of the Cyanogen Bromide Fragment of Fc Containing the Interchain Disulfide Bonds. Digestion with CNBr was carried out essentially as described by Press *et al.* (1966). After lyophilization to stop the reaction, the digest was taken up in 2 ml of 0.05 M acetic acid. A small amount of precipitate was removed and the supernatant material, representing 81% of the initial absorbancy units at 278 nm, was applied to a 1.5 × 60 cm column of Sephadex G-75 in 0.05 M acetic acid. The effluent was monitored by reading the optical density at 278 and 225 nm. The most retarded peak was identified by amino acid analysis to be the C-terminal fragment of the γ1 chain (Press *et al.*, 1966). The next to last peak (225 nm) was characterized by a very low absorption at 278 nm. This material was pooled and purified further by gel filtration with Sephadex G-50 in 0.02 M NH_4OH. Amino acid analysis indicated that it corresponded to the fragment 2a′′ described by Piggot and Press (1967), which is the fragment containing the two interchain disulfide bonds of Fc.

Antisera. Antisera to Gil IgG, Fab, and Fc, and to pooled human serum were prepared by immunizing rabbits with 2–5 mg of protein or 0.5 ml of serum emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Rabbit antiserum to Fab fragments from pooled human immunoglobulin was the gift of Dr. C. Alper.

Electrophoresis in Polyacrylamide Gels. Electrophoresis in polyacrylamide gels containing SDS was based on the method described by Shapiro *et al.* (1967). Gels contained 10% (w/v) acrylamide (Eastman), 0.27% (w/v) *N,N′*-methylenebisacrylamide (Eastman), 0.1% SDS (Matheson Coleman & Bell), and 0.05 M sodium phosphate buffer (pH 7.0). Polymerization was with 0.05% *N,N,N′,N′*-tetramethylethylenediamine (Eastman) and 0.1% ammonium persulfate. Most of the samples were "pretreated" by making them 1% in SDS and either heating to 60° for 1 hr or dialyzing at 37° for 12–17 hr against 0.1% SDS–0.01 M sodium phosphate (pH 7.0). Electrophoresis was for 3–4 hr at 6 V/cm in buffer containing 0.05 M sodium phosphate and 0.1% SDS. After the run, each gel was placed into a stoppered perforated plastic tube. These tubes were stirred at 4° for about 17 hr in a solution of 0.025% Coomassie brilliant blue R250 (Colab, Glenwood, Ill.) in 50% methanol–5% acetic acid. The gels were destained in 10% methanol–10% acetic acid for about 24 hr at 37° and stored in 7% acetic acid.

Electrophoresis in nondissociating conditions was carried out in a similar manner to that described above except that SDS was omitted and the buffer was 0.1 M sodium acetate (pH 4.0). The concentration of acrylamide was 7.5% and that of methylenebisacrylamide was 0.20%. For polymerization the concentration of *N,N,N′,N′*-tetramethylethylenediamine was increased to 0.2%. The samples (without "pretreatment") were applied directly to the gels and electrophoresis was carried out for about 3 hr at 13 V/cm in 0.1 M sodium acetate buffer (pH 4.0).

To measure ^{203}Hg in gels 1-mm slices were placed into

[2] Abbreviations used are: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Fc-Hg, mercury derivative of Fc; Fab-Hg, mercury derivative of Fab; Fc-RA, partially reduced alkylated Fc; Fab-RA, partially reduced alkylated Fab; IgG-RA, partially reduced alkylated IgG.

plastic tubes and radioactivity was measured in a γ counter. Alternatively, a variation of the procedure described by Choules and Zimm (1965) was used. Gels containing the cross-linking reagent ethylene diacrylate (K & K Laboratories, Plainview, N. Y.) were sliced, hydrolyzed in concentrated NH_4OH, and counted in a toluene-based scintillation fluid containing Triton X-100 (Rohm and Haas, Philadelphia, Pa.).

Radioactive Reagents and Measurements. $^{203}Hg(NO_3)_2$ was obtained from Cambridge Nuclear Corp., Cambridge, Mass., New England Nuclear Corp., Boston, Mass., or as a standard from the Nuclear Science and Engineering Corp., Pittsburgh, Pa. The spectrum of each shipment was checked in a γ counter (Packard Instrument Co.) and corresponded to that for ^{203}Hg with an energy peak at 0.28 MeV. Stock solutions were prepared by diluting the radioactive material with a volume of a carefully prepared solution of $HgCl_2$ and making the final solution 0.1 M in sodium acetate buffer (pH 4.0). This material will be referred to as $^{203}Hg^{2+}$. The specific activity of the stock solutions ranged from 0.08 to 6 mCi per mmole $^{203}Hg^{2+}$. All subsequent radioactivity measurements were related to the initial determination by using a correction factor for the half-life of ^{203}Hg, 47 days.

[1-^{14}C]Iodoacetic acid (New England Nuclear Corp.) was mixed with recrystallized nonradioactive iodoacetic acid and recrystallized three times from ether–petroleum ether. The specific activity of the product, measured in a liquid scintillation spectrometer (Packard Model 3320), was 2.86 μCi/mmole. [1-^{14}C]Iodoacetamide (New England Nuclear Corp.) was mixed with recrystallized nonradioactive iodoacetamide and recrystallized three times from H_2O. The specific activity of the product in two preparations was 42.5 and 83.0 μCi per mmole. Stock solutions of [1-^{14}C]iodoacetamide in H_2O or 0.1 M Tris-chloride (pH 8.0) were stored frozen in the dark and were found to be stable for several months.

Amino Acid Analysis. Acid hydrolysis was carried out at 110° for 20 hr in 6 N HCl under vacuum. The hydrolyzed samples were treated with pyridine–acetate buffer (pH 6.5) to convert homoserine lactone to homoserine (Ambler, 1965). The pyridine–acetate was removed by evaporation; the samples were taken up in 0.2 M sodium citrate buffer (pH 2.2) and immediately added to the 50-cm column of the analyzer. Quantitative analyses were carried out according to the method of Spackman *et al.* (1958) with a Beckman Model 120B amino acid analyzer. To resolve homoserine, the temperature of the run was maintained at 45° for the first 35 min and then increased to 55°. Homoserine was eluted between serine and glutamic acid. Values for half-cystine were determined after carboxymethylation as *S*-carboxymethylcysteine or after performic acid oxidation as cysteic acid.

Concentration Measurements. Protein concentrations were based on optical density measurements and nitrogen determinations. The latter were obtained with a Kjeldahl apparatus by Mrs. S. Luck and Dr. S. Lowey, Harvard Medical School. The weight fraction of nitrogen in Fc was taken as 16.5% based on the amino acid composition of the Eu γ1 Fc fragment (Edelman, 1970). The weight fraction of nitrogen in Fab was assumed to be 16.4% based on the composition of the New λ chain (Langer *et al.*, 1968) and the Fd portion of the Daw γ1 chain (Press and Hogg, 1970). The absorbance of the Gil Fc fragment at 278 nm and at 250 nm was the same in 0.1 M sodium acetate buffer (pH 4.0) as in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.2). The extinction coefficients at 278 nm of 1.00% (w/v) solutions of Gil IgG, Fab, and Fc in 0.15 M NaCl, 0.01 M potassium phosphate (pH 7.2) were 14.6, 15.0, and 14.4, respectively. These values were assumed for the proteins in several buffers in the pH range 4–8. The extinction coefficients of the mercury derivatives of Fab and Fc were assumed to be the same as those for the native proteins. (As will be discussed later a slight increase in optical density may accompany the formation of mercury–sulfur bonds, but the change at 278 nm is very small; *e.g.*, based on data obtained with model compounds, the replacement of both interchain disulfide bonds in Fc with bonds of the type S–Hg–S would increase the optical density at 278 nm by about 1%.) To obtain molar concentrations, the molecular weight of Fab was assumed to be 47,000 based on the composition of the New λ chain and the Fd portion of the Daw γ1 chain. The molecular weight of Fc was taken as 50,000 based on a calculated molecular weight (neglecting carbohydrate) of 49,900 for the Eu γ1 Fc fragment.

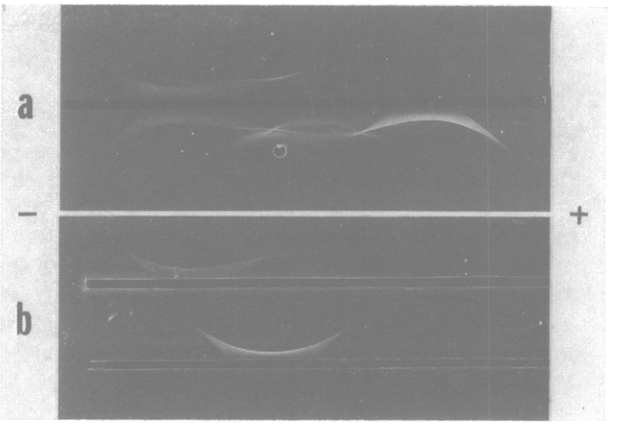


FIGURE 1: Immunoelectrophoresis of Gil proteins. (a) Upper well, Gil IgG 46 mg/ml; lower well, Gil serum; trough, antiserum to pooled human serum. (b) Uppermost and lowest well, Gil Fab 10 mg/ml; center well, Gil Fc 10 mg/ml; upper trough, antiserum to Gil Fab; lower trough, antiserum to Gil Fc. Electrophoresis was for 70 min at 300 V in 0.05 M sodium barbital buffer (pH 8.6).

Other Materials and Methods. Molecular models were constructed with CPK atomic models obtained from the Ealing Corp., Cambridge, Mass. Bence-Jones proteins of types K and L were donated by Dr. C. Baglioni, M.I.T. Double diffusion and immunoelectrophoresis were carried out in 1% agar (Ionagar No. 2, Colab) on microscope slides using apparatus of the Gelman Instrument Co. (Ann Arbor, Mich.). Zone electrophoresis on strips of cellulose acetate in 0.075 M barbital buffer pH 8.6 was done with the Phoroslide electrophoresis system (Millipore Corp., Bedford, Mass.). Sedimentation velocity experiments were made in a Spinco Model E ultracentrifuge equipped with absorption optics and operated at 59780 rpm and 22°. Fc and the mercury derivative of Fc were run simultaneously using a 1° wedge cell with quartz windows.

Results

Characterization of Gil Myeloma Protein and Its Fab and Fc Fragments. The preparation of myeloma protein obtained by chromatography with DEAE-cellulose moved as a single component when examined at a concentration of 46 mg/ml by zone electrophoresis on strips of cellulose acetate. Immunoelectrophoresis of the preparation with an antiserum to normal human serum showed a major cathodal precipitin arc with a characteristic "tail" and a minor cathodal band. The tail and the minor band were only detected at antigen concentrations greater than 10 mg/ml (Figure 1a). Double diffu-

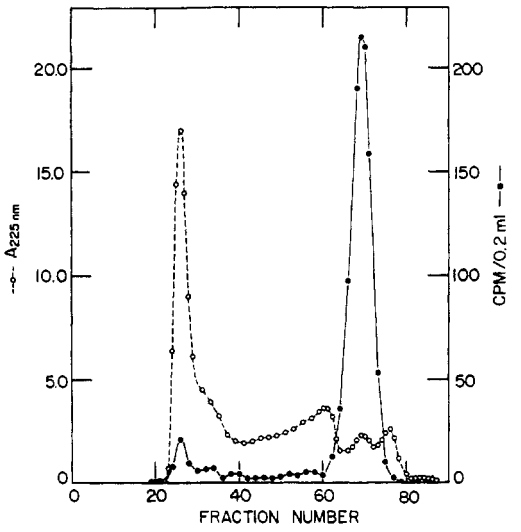


FIGURE 2: Gel filtration of a CNBr digest of Fc. The protein was partially reduced and alkylated with [1-^{14}C]iodoacetic acid before digestion with CNBr. The digest (24 mg) was added to a 1.5 × 60 cm column of Sephadex G-75 in 0.05 M acetic acid and fractions of 1.3 ml were collected.

sion in agar with antiserum against pooled human Fab fragments revealed that the major component was antigenically deficient and the minor component antigenically identical with pooled human (Red Cross) immunoglobulin. Evidently, the major component was the myeloma protein; since it was estimated to represent at least 98% of the total protein obtained from the DEAE-cellulose column, no further purification was attempted. The preparation will be referred to henceforth as "Gil IgG." Genetic typing of the preparation, carried out by Dr. H. Borel, Harvard Medical School, showed it to be Gm (4). A rabbit antiserum against the Fab fragment of Gil IgG reacted with a Bence-Jones protein, type L, and not with a Bence-Jones protein, type K. Accordingly, Gil protein is IgG1, Gm (4), type L.

The Fab fragment was obtained by chromatography of a papain digest of Gil IgG on DEAE-cellulose followed by gel filtration with Sephadex G-100. Analysis by electrophoresis on strips of cellulose acetate revealed a single major component and a second more slowly moving component representing about 5–10% of the total protein. A minor slow component was also apparent upon electrophoresis in polyacrylamide gels in acetate buffer at pH 4.0 (see later section, Figure 13a). However, when the Fab preparation was examined by electrophoresis in polyacrylamide gels containing SDS, only one strong band was seen together with a small amount of two faster moving components that were shown to be free light chain and Fd (see later section, Figure 11a). The minor slow component could be separated from the major component by gel filtration with Sephadex G-100. The two fractions obtained in this way were indistinguishable when analyzed by electrophoresis in SDS–polyacrylamide gels. It was concluded that the minor component was an aggregate of Fab that formed during storage of the protein. The Gil Fab fragment at all concentrations tested (up to 41 mg/ml) did not react in immunoelectrophoresis with an antiserum directed against Gil Fc fragment (Figure 1b).

The Fc fragment was obtained from the same DEAE-cellulose column by elution with a gradient of NaCl. It was homogeneous by zone electrophoresis on cellulose acetate and also when analyzed by electrophoresis in polyacrylamide gels in acetate buffer at pH 4.0 or in buffer containing SDS. At concentrations below 20 mg/ml the Fc preparation did not react with an antiserum to Gil Fab (Figure 1b). At greater concentrations a faint band, identified as Gil Fab, was detected. The contamination with Fab was estimated to be less than 2%.

Preparation of Mercury Derivative of Fc. SELECTIVE REDUCTION OF INTERCHAIN DISULFIDE BONDS IN FC. In order to introduce mercury specifically into the two interchain disulfide bridges of the Fc fragment it was necessary to establish conditions for the complete but selective reduction of these two bonds. The Fc fragment at a concentration of 24 mg/ml was incubated at 37° in 5 mM dithiothreitol–0.1 M Tris-chloride (pH 8.0). At the end of 1 hr the solution was made 14 mM in [1-^{14}C]iodoacetic acid, incubated for 30 min at room temperature, and then dialyzed exhaustively against 0.05 M Tris-chloride (pH 8.0). From the specific activity of the modified protein it was determined that 3.8 moles of [1-^{14}C]iodoacetate had reacted with each mole of Fc fragment. When this material was analyzed by electrophoresis in polyacrylamide gels containing SDS or by gel filtration in 1 M acetic acid, the virtually complete conversion of Fc into a subunit of lower molecular weight was demonstrated.

To confirm that only the interchain disulfide bonds had been cleaved, it remained to show that the alkylating agent was attached exclusively to sulfhydryl groups derived from the two interchain S–S bridges. The reduced carboxymethylated Fc fragment was digested with CNBr and the digest was fractionated on a column of Sephadex G-75 equilibrated with 0.05 M acetic acid. As shown in Figure 2 almost all of the radioactivity was confined to a single peak. This material was pooled and rerun on a column of Sephadex G-50 in the same solvent. Amino acid analysis of the resulting material indicated that it corresponded to the CNBr fragment of Fc that had previously been shown to contain the two disulfide bonds joining the heavy chains in human IgG1 (Steiner and Porter, 1967). Since this fragment contains no other residues of half-cystine, it was concluded that these conditions of reduction resulted in the cleavage of only the two interchain disulfide bonds in Fc.

TITRATION OF PARTIALLY REDUCED FC WITH $^{203}Hg^{2+}$. The interchain disulfide bridges in the Fc fragment were reduced by incubating 17 mg of the protein in 0.7 ml of 5 mM dithiothreitol at pH 8.0. At the end of 1 hr 0.1 ml of the reaction mixture was removed and alkylated for 1 hr at room temperature with 1.4 μmoles of [1-^{14}C]iodoacetamide. The specific activity of the resulting protein after dialysis indicated 4.0 moles of [1-^{14}C]iodoacetamide/mole of Fc fragment, corresponding to the reduction of 2.0 disulfide bonds. The pH of the remaining reaction mixture (0.6 ml) containing protein and DTT was lowered to 4.0 with 2 M acetic acid and this material was applied to a 0.8 × 18 cm column of Sephadex G-25 (fine) equilibrated with 0.1 M sodium acetate buffer (pH 4.0). Elution was carried out rapidly with the same buffer under N_2 pressure. The fraction containing the protein (determined by previous calibration of the column) was obtained in less than 5 min. Aliquots of this were immediately added to a series of test tubes containing various amounts of $^{203}Hg^{2+}$ in 0.1 M sodium acetate buffer (pH 4.0). The final concentration of protein in these tubes was approximately 1 mg/ml. After incubation at room temperature for 30 min, each fraction was dialyzed against 200 ml of the same buffer for 36 hr. The samples were then removed from the dialysis bags and centrifuged to ensure optical clarity although usually there was no visible

TABLE I: Titration of Partially Reduced Fc with $^{203}Hg^{2+}$.

Sample	$^{203}Hg^{2+}$ Added (Moles of Hg/Mole of Fc)[a]	$^{203}Hg^{2+}$ Bound to Protein (Moles of Hg/Mole of Fc) Dialysis			OD Ratio of Protein $A_{278\ nm}/A_{250\ nm}$ Dialysis			$^{203}Hg^{2+}$ on Dialysis Bag (nmoles) Dialysis		
		1	2	3	1	2	3	1	2	3
1	0	0	0	0	2.86	2.93	2.92	0	0	0
2	0.42	0.42	0.42	0.41	2.80	2.85	2.84	0.12	0.19	0.14
3	0.84	0.84	0.83	0.82	2.77	2.76	2.75	0.27	0.31	0.26
4	1.26	1.22	1.21	1.20	2.71	2.65	2.73	0.51	0.50	0.31
5	1.68	1.65	1.65	1.63	2.56	2.62	2.62	0.49	0.63	0.53
6	2.10	1.90	1.88	1.86	2.58	2.58	2.56	3.6	0.99	0.44
7	2.94	2.32	1.98	1.95	2.28	2.52	2.58	12.7	6.0	0.86
8	3.93	2.53	2.10	1.99	2.13	2.46	2.54	27.8	7.6	1.8

[a] At the beginning of the experiment, the specific activity of the preparation of $^{203}Hg^{2+}$ was 7.7×10^3 cpm/nmole. The amount of Fc in each sample was 22.2 nmoles.

precipitate. The optical density of the samples at 340, 278, and 250 nm was determined with the dialysate as blank. (The absorbance at 340 nm was usually less than 0.5% of that at 278 nm indicating that there was no significant light scattering in the samples.) In addition, the radioactivity of the samples and dialysates was measured by counting 0.5-ml aliquots in the γ counter. The dialysis bags were washed with dilute acetic acid and also counted. The samples were then subjected to two more such cycles of dialysis and analysis.

The results of the titration of partially reduced Fc with $^{203}Hg^{2+}$ are summarized in Table I and Figure 3. The mercuric ion added was completely bound by the protein until an end point was reached at 1.96 moles of $^{203}Hg^{2+}$ added per mole of Fc. Beyond this end point some additional Hg^{2+} could be bound, but this was gradually removed by the successive dialyses. It is noteworthy that all of the mercuric ion not complexed to the protein was bound to the dialysis tubing. No $^{203}Hg^{2+}$ appeared in the dialysate. Indeed, after the first step of dialysis, the sum of mercury bound to the protein and that adhering to the dialysis tubing corresponded quite closely to the amount of $^{203}Hg^{2+}$ added. Thus, the end point of the titration was also indicated by the appearance of radioactively labeled Hg^{2+} on the dialysis bags (Figure 3). Pretreatment of the dialysis casing by heating in dilute solutions of EDTA had no apparent effect on its propensity to bind mercuric ion.

Two additional titration experiments were carried out with different lots of $^{203}Hg^{2+}$. The amounts of mercuric ion added covered the range up to a fivefold molar excess with respect to protein. The results were essentially the same as those summarized in Table I and Figure 3 with end points at 1.88 and 1.98 moles of $^{203}Hg^{2+}$ per mole of Fc.

To confirm that reduction was required for binding mercuric ion, the native unreduced Fc fragment was reacted with $^{203}Hg^{2+}$. In this case, 0.06 mole of Hg^{2+} was bound per mole of protein. Unreduced Fc also took up only 0.04 mole of [1-^{14}C]iodoacetamide/mole of protein. As an additional control, a derivative of Fc was prepared in which those cystine residues that are reduced in 5 mM dithiothreitol were blocked by reaction with [^{12}C]iodoacetamide. This derivative was subjected to a second step of reduction in 5 mM dithiothreitol and then reacted with $^{203}Hg^{2+}$ under the usual conditions. Only 0.15 mole of Hg^{2+} was now taken up per mole of protein indicating that the groups that usually react with mercuric ion were no longer available.

The reaction of the partially reduced Fc fragment with Hg^{2+} could also be followed by determining the ratio of its absorbance at 278 nm to that at 250 nm. For native Fc this ratio is 2.9 ± 0.05. As increasing amounts of mercuric ion were bound to the protein, the ratio decreased (Table I). At the "plateau level" with about 2.0 moles of Hg^{2+} bound per mole of Fc, the ratio of optical density at 278 nm to that at 250 nm was 2.55 ± 0.05. If additional Hg^{2+} was bound, the ratio was less than 2.50 (Table I, samples 7 and 8, dialysis 1). These changes in optical density are probably related to the absorbance of bonds of the type S–Hg–S and –S–Hg^+. The spectral properties of such bonds have been studied by Steinberg and Sperling (1967). We have also investigated the changes in the optical density of a model compound, cysteine, when it is reacted with various amounts of mercuric ion in 0.1 M sodium acetate buffer (pH 4.0). At a molar ratio of Cys:Hg^{2+} of 2:1 a product of the type RS–Hg–SR (R = cysteine) is presumably

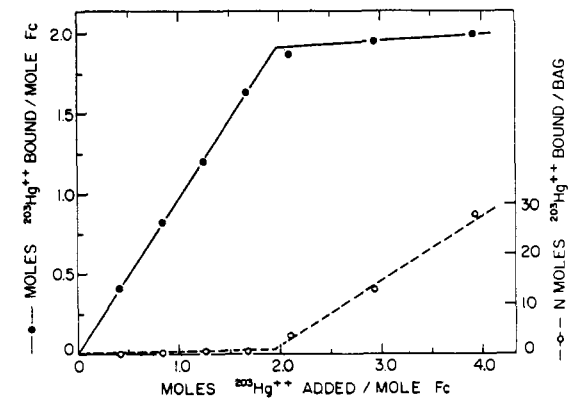


FIGURE 3: Titration of partially reduced Fc with $^{203}Hg^{2+}$. Samples (1.11 mg) were added to various amounts of $^{203}Hg^{2+}$ in 0.1 M sodium acetate (pH 4.0) and then dialyzed three times to remove unbound or loosely bound mercury. Mercuric ion not tightly bound to the protein adhered to the dialysis tubing. (●) Moles of $^{203}Hg^{2+}$ bound per mole of Fc after the third step of dialysis. (○) Nanomoles of $^{203}Hg^{2+}$ bound by each dialysis bag after the first step of dialysis. (See also Table I.)

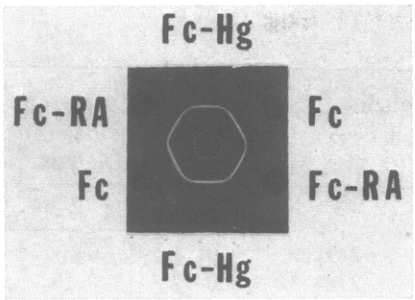


FIGURE 4: Immunodiffusion of Fc, Fc-Hg, and Fc-RA. The center well contained antiserum to native Fc. The concentration of each antigen was 0.2 mg/ml. The agar was made up in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.2).

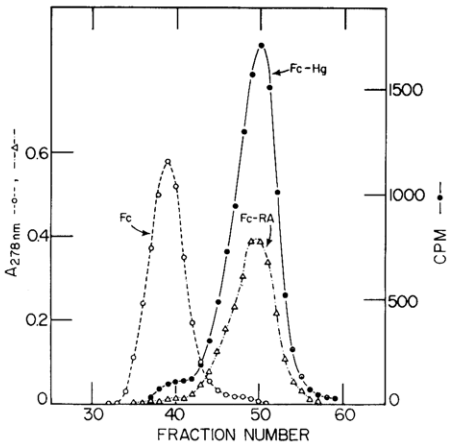


FIGURE 5: Gel filtration of Fc, Fc-Hg, and Fc-RA on a 1.1 × 60 cm column of Sephadex G-100 in 1 M acetic acid. Effluent fractions were 0.5 ml. Fc (1.8 mg) was chromatographed alone. Fc-Hg (0.1 mg, 13,000 cpm) and Fc-RA (1.3 mg, no radioactive label) were mixed and chromatographed together; the position of Fc-Hg was established from the radioactivity of the whole effluent fractions, and that of Fc-RA was obtained from the optical density profile.

formed and this is accompanied by a very slight increase in absorbance at 278 nm and a more pronounced increase at 250 nm. As additional Hg^{2+} is added there is a further increase in absorbance at both wavelengths with the presumptive formation of complexes of the type RS–Hg^{+}. The formation of mercury–sulfur bonds in the protein was also accompanied by small changes in optical density. Although the magnitude of these changes could not necessarily be predicted from the extinction coefficients of the model compounds, the ratio of absorbance at 278 nm to that at 250 nm was a useful empirical guide for following the reaction of the protein with mercuric ion.

We concluded from these experiments that the partially reduced Fc fragment can bind tightly approximately 2 moles of $^{203}Hg^{2+}$/mole of protein, corresponding to the two labile interchain disulfide bonds that are cleaved by gentle reduction. This ligand is probably found in bonds of the type S–Hg–S. The partially reduced protein may take up additional Hg^{2+} but this "extra" ligand is easily removed, for example by competitive binding to sites on dialysis tubing. Even some of the "tightly bound" mercuric ion may be gradually lost by dialysis (Table I, samples 2–5), but the amount removed in this way is a small fraction of the total.

BATCH PREPARATION OF Fc-Hg. Larger quantities of the mercury derivative of Fc were prepared by a slight modification of the procedure described above. The effluent from the G-25 column containing the reduced protein was collected directly, with stirring, into an approximately three- to fourfold molar excess of $^{203}Hg^{2+}$ in 0.1 M sodium acetate buffer (pH 4.0). The volume was adjusted so that the final protein concentration was about 0.1%. The sample was repeatedly dialyzed until the specific activity of the derivative had stabilized at the level of about 1.95 ± 0.05 moles of $^{203}Hg^{2+}$/mole of protein. The amount of Hg^{2+} adherent to the dialysis bag was then about 2–3% of that in the sample. This usually required three to four changes of dialysis bag and was largely unaffected by variations in the length of each period of dialysis. The preparations of Fc-Hg were stored at 4° in 0.1 M sodium acetate buffer and were stable for months.

Alternatively, the excess mercuric ion was removed by gel filtration with Sephadex G-25. The derivative appeared in the void volume and the specific activity indicated that it contained 1.95 moles of $^{203}Hg^{2+}$/mole of Fc. Most of the free or loosely bound Hg^{2+} adhered to the Sephadex particles and did not appear in the effluent. A second step of gel filtration did not decrease the specific activity of the derivative.

Properties of Fc-Hg. The mercury derivative of Fc sedimented as a single symmetrical peak in the ultracentrifuge. At a concentration of 0.8 mg/ml in 0.1 M sodium acetate–0.1 M NaCl (pH 4.0) its sedimentation coefficient ($s_{20,w}$) was 3.76 ± 0.05 S; for native Fc $s_{20,w}$ was 3.69 ± 0.05 S. Fc and the mercury derivative were eluted in the same position from a column of Sephadex G-100 equilibrated in 0.1 M sodium acetate (pH 4.0). Indeed, when the native protein was mixed with a tracer amount of derivative, the optical density and radioactivity elution profiles were identical. In addition, the native Fc fragment, reduced alkylated Fc, and the mercury derivative were indistinguishable when analyzed by electrophoresis in polyacrylamide gels in 0.1 M sodium acetate buffer (pH 4.0). Finally, as shown in Figure 4, the three proteins were identical in their reactivity with an antiserum prepared against native Fc.

Crystallization of Fc-Hg. When concentrated solutions (*i.e.*, 0.5–1.0%) of Fc-Hg were dialyzed against dilute neutral buffers (*e.g.*, 1 mM sodium phosphate, pH 7.0) rodlike birefringent crystals of average dimensions 0.1 × 0.5 mm were formed. The crystals were soluble in 0.3 M NaCl–0.02 M Tris-chloride (pH 8.0) or in 0.1 M sodium acetate (pH 4.0). The specific activity of the crystals was identical with that of the mother liquor indicating that the ^{203}Hg was retained by the protein after crystallization. X-Ray diffraction patterns of crystals of Fc-Hg were obtained and analyzed by Dr. R. Poljak, Johns Hopkins University. The unit cell dimensions were very similar to those of native Fc, but the symmetry was that of space group $C222_1$ rather than $P2_12_12_1$ for native Fc (Humphrey, 1967). Thus, the crystals of Fc-Hg were not isomorphous with those of the native molecule. However, it should be noted that the molecular packing of native Fc, although strictly described by space group $P2_12_12_1$, is very close to $C222_1$ (Goldstein *et al.*, 1968).

Subunit Structure of Fc-Hg. The difference in the space groups of crystals of Fc-Hg and native Fc suggested that the formation of the mercury derivative may have been accompanied by some degree of perturbation in molecular structure. Perhaps the addition of Hg^{2+} induced a rearrangement in the disulfide bonding. Native Fc derived from IgG1 is a dimer, cross-linked by two closely spaced disulfide bonds. If these disulfide bridges are cleaved, for example by reduction and alkylation, the two subunits remain associated by noncova-

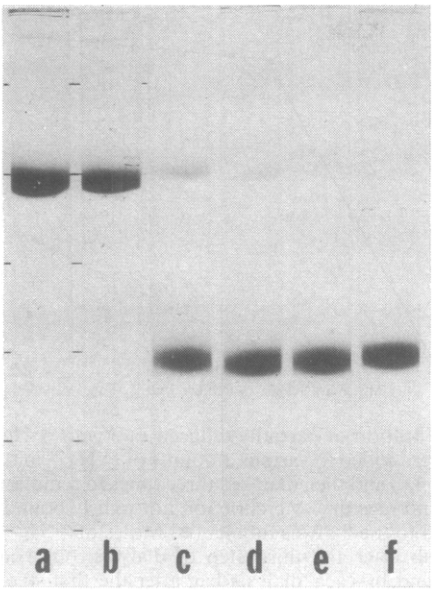


FIGURE 6: Electrophoresis of Fc, Fc-Hg, and Fc-RA in 10% polyacrylamide gels–0.1% SDS–0.05 M sodium phosphate (pH 7.0). (a) Fc; (b) native (unreduced) Fc that had been treated with $^{203}Hg^{2+}$; (c and d) two preparations of Fc-Hg; (e and f) two preparations of Fc-RA. Samples a and e were in 0.1 M Tris-chloride (pH 8.0) and were made 1% in SDS and heated to 60° for 1 hr. Sample c was in 0.1 M sodium acetate (pH 4.0) and was also "pretreated" with 1% SDS and heat. Samples b, d, and f were made 1% in SDS and then dialyzed at 37° for 9 hr against 0.1% SDS–0.01 M sodium phosphate (pH 7.0).

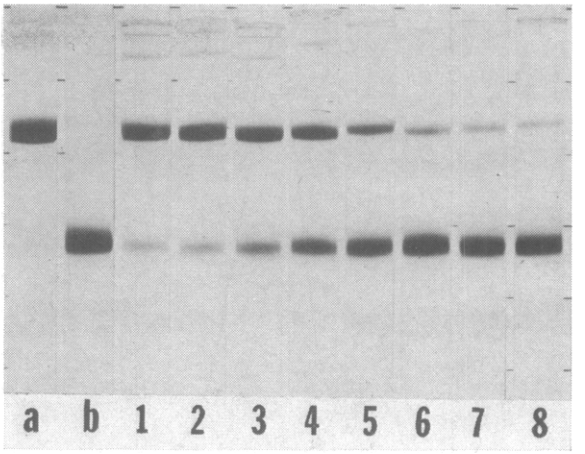


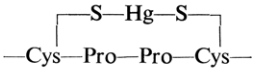
FIGURE 7: Electrophoresis of partially reduced Fc titrated with $^{203}Hg^{2+}$; 10% polyacrylamide gels contained 0.1% SDS–0.05 M sodium phosphate (pH 7.0). (a) Native Fc; (b) Fc-RA; 1–8 correspond to the samples after the third dialysis step in the titration experiment summarized in Table I and Figure 3. All samples were in 0.1 M sodium acetate buffer (pH 4.0) and were made 1% in SDS and heated to 60° for 1 hr.

lent forces (*e.g.*, see Inman and Nisonoff, 1966). Such dimers are indistinguishable in most properties from the native molecule but can be dissociated into monomers in certain solvents. Fc-Hg presumably differs from native Fc in that the interchain disulfide bonds have been converted by reduction and reaction with mercuric ion into bonds of the type S–Hg–S. To determine whether or not Fc-Hg is a dimer cross-linked by such mercury–sulfur bonds, it was necessary to investigate its subunit structure in dissociating solvents.

The noncovalent interactions between immunoglobulin subunits are disrupted in 1 M acetic or propionic acid (Fleischman *et al.*, 1962). Accordingly, native Fc, Fc-Hg, and reduced alkylated Fc were each subjected to gel filtration on a column of Sephadex G-100 equilibrated in 1 M acetic acid. As shown in Figure 5, the mercury derivative was eluted in the monomer position of reduced alkylated Fc, not in the dimer position of native Fc.

The subunit structure of Fc-Hg was also evaluated in another dissociating solvent, sodium dodecyl sulfate. The electrophoretic migration of native Fc, Fc-Hg, and reduced alkylated Fc in polyacrylamide gels containing SDS is shown in Figure 6. Again, the mercury derivative had the mobility of the monomer. In agreement with the results of staining, counting of gel slices indicated that approximately 98% of the radioactivity recovered was in the monomer position. Figure 7 demonstrates that the conversion of native Fc into Fc-Hg could be followed by analyzing the various samples in the titration experiment described previously (see Table I and Figure 3). As the end point was approached, there was a gradual transition from the dimer to the monomer form. Despite the complete reduction of the interchain disulfide bonds in this experiment, when less than an equivalent amount of Hg^{2+} was added, some of the Fc behaved as a dimer (*e.g.*, Figure 7, sample 1). Therefore, during the incubation or dialysis at pH 4, interchain disulfide bridges must have formed between cysteine residues not blocked by Hg^{2+}, with re-formation of the Fc dimer.

These experiments demonstrate that at least in dissociating solvents such as acetic acid or SDS the mercury derivative of Fc is a monomer. The amino acid sequence near the two interchain disulfide bonds in Fc may account for this. These two disulfide brdiges are separated by only two residues of proline (Steiner and Porter, 1967; Frangione and Milstein, 1967; Gall *et al.*, 1968); *i.e.*, the sequence is -Cys-Pro-Pro-Cys-. A space-filling molecular model of this sequence shows that the separation of the two residues of cysteine is such that it is possible to fit a mercury atom exactly between them forming an intrachain loop, thus



The formation of such a loop could account for the apparent conversion, by mercuric ion, of the native dimer into a monomer. Moreover, the rearrangement in disulfide bonding might sufficiently affect the packing of the Fc subunits to bring about changes in crystallization. It cannot, however, be concluded from these results that Fc-Hg is necessarily a monomer also in nondissociating solvents. It is possible that Fc-Hg in its native state is a dimer cross-linked by bridges of the type S–Hg–S and that it rearranges into the monomer only in dissociating solvents (see Discussion).

Mercury Derivative of the Interchain Disulfide Peptide. To obtain further information on the conformational possibilities for the mercury derivative of Fc in nondissociating solvents, studies with a model compound were carried out. A suitable compound is the CNBr fragment of Fc that contains both interchain disulfide bonds but no other residues of cysteine. This peptide is a dimer of the 28 N-terminal residues of Fc and it was isolated with the two interchain disulfide bonds intact. The peptide (0.26 μmole of dimer) was reduced by incubation at 37° for 1 hr in 1.1 ml of 5 mM dithiothreitol–0.1 M Tris-chloride (pH 8.0). An aliquot of the reaction mixture (0.3 ml) was treated with 15 mM [1-^{14}C]iodoacetamide, incubated at 37° for 1 hr, diluted fivefold with 0.1 M sodium acetate buffer

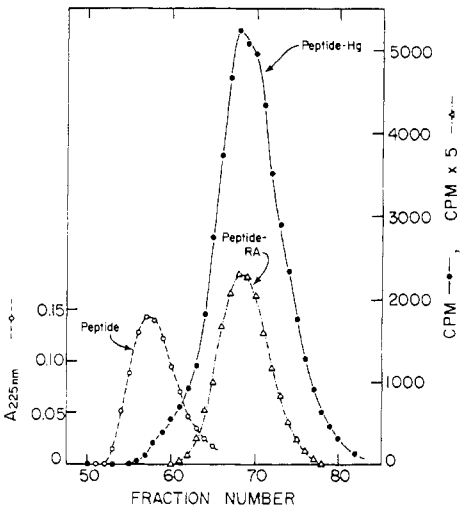


FIGURE 8: Gel filtration of interchain disulfide peptide (peptide), mercury derivative of peptide (peptide–Hg), and reduced, alkylated peptide (peptide–RA) on a 1.1 × 60 cm column of Sephadex G-50 in 0.1 M sodium acetate (pH 4.0). The samples were chromatographed one at a time and fractions of 0.5 ml were collected. For the peptide, 0.025 μmole of unreduced dimer was mixed with 0.1 μmole of $^{203}Hg^{2+}$ and added to the column. No radioactivity was contained in the effluent fractions and only the optical density profile is shown on the graph. For peptide–Hg, approximately 0.19 μmole (dimer) of the mercury derivative of the reduced peptide was added to the column. Each fraction (0.5 ml) was counted and the optical density at 225 nm was determined. The radioactivity and optical density profiles coincided and only the radioactivity values are shown on the graph. For peptide RA 0.07 μmole (dimer) of reduced alkylated peptide was added to the column. The optical density at 225 nm was determined and 0.1 ml of each fraction was counted. The two elution profiles coincided and only the radioactivity values are shown.

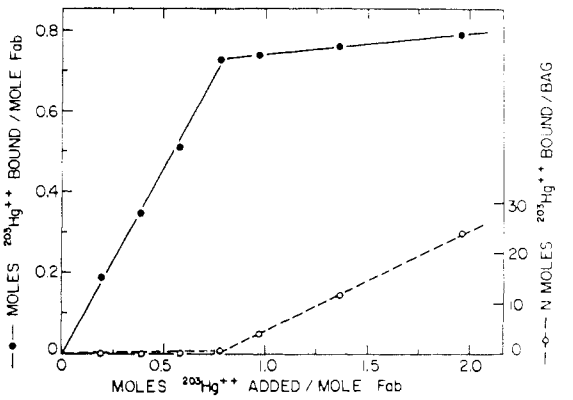


FIGURE 9: Titration of partially reduced Fab with $^{203}Hg^{2+}$. Samples (1.13 mg) were added to various amounts of $^{203}Hg^{2+}$ in 0.1 M sodium acetate (pH 4.0) and then dialyzed three times to remove unbound or loosely bound mercury. Mercuric ion not tightly bound to the protein adhered to the dialysis tubing. (●) Moles of $^{203}Hg^{2+}$ bound per mole of Fab after the third step of dialysis. (○) Nanomoles of $^{203}Hg^{2+}$ bound by each dialysis bag after the first step of dialysis. (See also Table II.)

(pH 4.0), and stored for 4 hr at 4°. The remainder of the reaction mixture (0.8 ml) was adjusted to pH 4.0 with 2 M acetic acid, added to a 0.9 × 16 cm column of Sephadex G-15 equilibrated with 0.1 M sodium acetate buffer (pH 4.0), and eluted rapidly with N_2 pressure. The eluate fraction containing the reduced peptide was collected directly, with stirring, into 0.75 μmole of $^{203}Hg^{2+}$ in 0.1 M sodium acetate buffer (pH 4.0), and incubated for 1 hr at room temperature. The reaction mixture, now containing peptide and mercuric ion, was added to a column of Sephadex G-50 equilibrated with the same buffer. When the elution of the mercury-containing peptide was completed, the reduced alkylated peptide that had been stored at 4° was added to the same column. Finally a sample of unreduced peptide that had been treated with $^{203}Hg^{2+}$ was added to the column.

The elution patterns of these peptides are shown in Figure 8. The native or untreated peptide was eluted well ahead of the reduced alkylated peptide and these two peaks mark the dimer and monomer positions, respectively. Evidently, a pair of these unlinked 28 residue chains is not held together by noncovalent forces in acetate buffer (pH 4.0). The mercury derivative of the peptide was eluted in the same position as the reduced alkylated peptide indicating that it too is a monomer. Analysis of the derivative for radioactivity and amino acid content showed that each mole of peptide monomer contained 0.90 mole of mercuric ion, or almost 2 moles of $^{203}Hg^{2+}$/mole of peptide dimer. The unreduced peptide did not bind any mercury. Thus, the extent of reaction with $^{203}Hg^{2+}$ was approximately the same for the peptide as for the intact Fc fragment. These results indicate that, at least in the case of the peptide, the intrachain loop can be formed in acetate buffer (pH 4.0).

Preparation of Mercury Derivative of Fab. The Fab fragment has only a single interchain disulfide bond bridging light chain and Fd. In contrast to Fc, there should be no possibility of forming an intrachain loop with Hg^{2+} and a pair of cysteine residues from the same chain, provided that only the interchain disulfide bond is cleaved. It was of interest therefore to determine whether the mercury derivative of Fab would form an interchain bridge of the type R–S–Hg–S–R′ where R and R′ are light chain and Fd.

SELECTIVE REDUCTION OF INTERCHAIN DISULFIDE BOND IN FAB. The Fab fragment at a concentration of 24 mg/ml was incubated in 5 mM dithiothreitol–0.1 M Tris-chloride (pH 8.0). At the end of 1 hr at 37° the solution was made 14 mM in [1-^{14}C]-iodoacetamide and incubated at room temperature for an additional hour. From the specific activity of the resulting protein after dialysis it was determined that 1.8 moles of iodoacetamide had reacted per mole of protein, corresponding to the cleavage of 0.90 disulfide bond. Analysis of the product by electrophoresis in SDS–polyacrylamide gels or by gel filtration in acetic acid indicated virtually complete conversion of Fab into its subunits, light chain and Fd. Thus, the disulfide bond that had been cleaved must have been the interchain bridge. Unreduced Fab bound only 0.04 mole of [1-^{14}C]iodoacetamide/mole of protein.

TITRATION OF PARTIALLY REDUCED FAB WITH $^{203}Hg^{2+}$. The preparation of the mercury derivative of Fab was analogous to that for Fc. The results of a titration of partially reduced Fab with $^{203}Hg^{2+}$ are summarized in Table II and Figure 9. The mercuric ion added was completely bound by the protein until an end point was reached at 0.80 mole of $^{203}Hg^{2+}$ added per mole of protein. Despite the addition of more ligand, almost no additional Hg^{2+} was bound. Again, the end point of the titration was also indicated by the appearance of radioactively labeled mercuric ion on the dialysis bags. Unreduced Fab fragment bound less than 0.03 mole of $^{203}Hg^{2+}$/mole of protein.

Evidently, partially reduced Fab reacted with somewhat less mercury (and also with less [1-^{14}C]iodoacetamide) per mole of interchain disulfide bond than did partially reduced

TABLE II: Titration of Partially Reduced Fab with $^{203}Hg^{2+}$.

Sample	$^{203}Hg^{2+}$ Added (Moles of Hg/ Mole of Fab)[a]	$^{203}Hg^{2+}$ Bound to Protein (Mole of Hg/Mole of Fab) Dialysis			OD Ratio of Protein $A_{278\ nm}/A_{250\ nm}$ Dialysis			$^{203}Hg^{2+}$ on Dialysis Bag (nmoles) Dialysis		
		1	2	3	1	2	3	1	2	3
1	0	0	0	0	3.10	3.12	3.06	0	0	0
2	0.19	0.19	0.19	0.19	3.09	3.10	3.12	0.14	0.09	0.07
3	0.39	0.39	0.38	0.35				0.14		
4	0.58	0.57	0.57	0.51	3.07	3.06	3.07	0.19	0.21	0.25
5	0.78	0.76	0.73	0.73	3.03	3.03	3.01	0.65	0.35	0.26
6	0.97	0.79	0.75	0.74	3.04	3.05	3.06	4.0	0.75	0.35
7	1.36	0.82	0.79	0.76	3.04	3.02	3.00	11.6	0.87	0.47
8	1.96	0.88	0.82	0.79	3.03	3.00	2.99	23.9	0.87	0.50

[a] At the beginning of the experiment, the specific activity of the preparation of $^{203}Hg^{2+}$ was 7.7×10^3 cpm/nmole. The amount of Fab in each sample was 24.0 nmoles.

Fc. One reason for this could be that the interchain cystine bridges in some of the Fab molecules may have become unavailable for reduction because of their prior cleavage and possible alkylation during the papain digestion. Indeed a small fraction (about 5%) of free light chains and Fd fragments could be detected in the Fab preparation by gel filtration in acetic acid or by electrophoresis in polyacrylamide gels containing SDS (see Figure 11a in later section).

Slight changes in the optical spectrum were noted when the partially reduced Fab fragment was reacted with Hg^{2+}. For native Fab the ratio of absorbance at 278 nm to that at 250 nm is 3.10 ± 0.05. As Hg^{2+} was bound to the protein, the ratio decreased to 3.00 ± 0.05 when 0.7–0.8 mole of mercuric ion was bound per mole of protein.

We concluded from these experiments that about 0.8 mole of Hg^{2+} could be bound by each mole of partially reduced Fab and that this ligand was associated with the disulfide bridge that normally joins the light chain and Fd subunits.

BATCH PREPARATION OF FAB-Hg. The mercury derivative of Fab was also prepared in larger quantities by collecting the partially reduced protein from the small Sephadex column directly into a solution of $^{203}Hg^{2+}$ in acetate buffer (pH 4.0), as in the batch method described for the preparation of Fc-Hg. Excess Hg^{2+} was removed either by dialysis or by gel filtration on a column of Sephadex G-25. Preparations of Fab-Hg contained 0.82 ± 0.05 mole of Hg^{2+}/mole of Fab. They were stored at 4° in 0.1 M sodium acetate buffer (pH 4.0) and were stable for months.

Properties of Fab-Hg. The Fab fragment and its radioactively labeled mercury derivative were eluted in the same position from a column of Sephadex G-100 in 0.1 M acetate buffer (pH 4.0). Native Fab and Fab-Hg were also indistinguishable upon electrophoresis in polyacrylamide gels in this buffer. The two proteins and a preparation of partially reduced alkylated Fab were antigenically identical in immunodiffusion with an antiserum prepared against native Fab.

Subunit Structure of Fab-Hg. Native Fab, Fab-Hg, and reduced alkylated Fab were each chromatographed on a column of Sephadex G-100 in 1 M acetic acid. The reduced alkylated protein was eluted in two approximately equal peaks, the first corresponding to Fd and the second to light chain. The mercury derivative was eluted as a skewed peak in the approximate position of native Fab but with a pronounced trailing shoulder (Figure 10).

Because of the apparent heterogeneity of Fab-Hg, the proteins were also analyzed by electrophoresis in polyacrylamide gels containing SDS. Native Fab (or Fab treated with iodoacetamide but not reduced) was composed of a single major component and a small amount of two closely spaced faster moving components (Figure 11a). The latter corresponded in mobility to the bands in reduced alkylated Fab (Figure 11b). The positions of light chain and Fd were established by comparing the components in reduced alkylated Fab with those in reduced alkylated Gil IgG (Figure 11b,e). The subunit common to both of these samples is light chain and the other major component in reduced Fab must be Fd. The

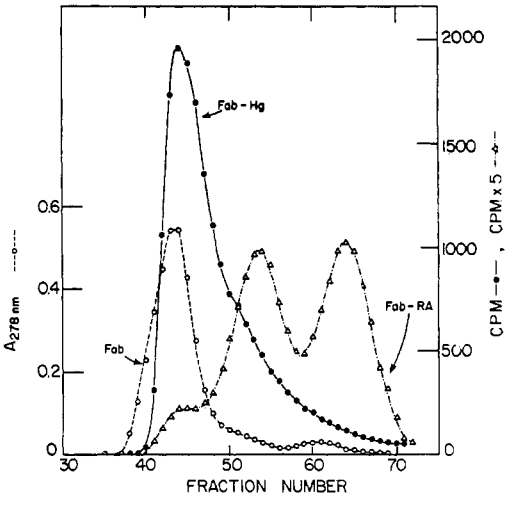


FIGURE 10: Gel filtration of Fab, Fab-Hg, and Fab-RA on a 1.1 × 60 cm column of Sephadex G-100 in 1 M acetic acid. Effluent fractions were 0.5 ml. The samples were chromatographed one at a time and the following amounts were used: Fab, 2 mg; Fab-Hg, 0.4 mg, 30,000 cpm; Fab-RA, 1.5 mg, 8000 cpm. In each case the optical density of the effluent fractions was determined, but on the graph only the radioactivity profiles for Fab-Hg (each fraction was counted in total) and Fab-RA (0.2 ml of each fraction was counted) are shown.

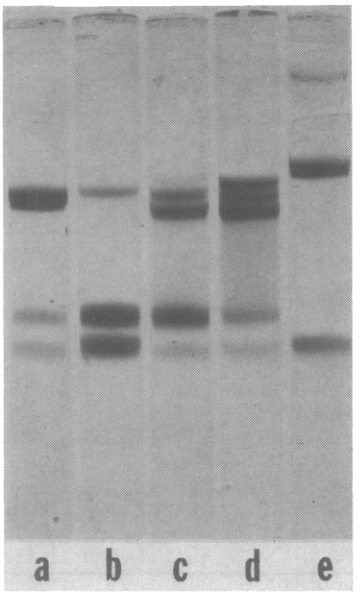


FIGURE 11: Electrophoresis of Fab, Fab-Hg, and Fab-RA in 10% polyacrylamide gels–0.1% SDS–0.05 M sodium phosphate (pH 7.0). (a) Fab (unreduced) that had been treated with [1-^{14}C]iodoacetamide; (b) Fab-RA; (c and d) two samples from the same preparation of Fab-Hg with different pretreatment; (e) IgG-RA. Samples a, b, c, and e were made 1% in SDS and dialyzed at 37° for 9 hr against 0.1% SDS–0.01 M sodium phosphate buffer (pH 7.0). Sample d was in 0.1 M sodium acetate (pH 4.0) and was made 1% in SDS and heated to 60° for 1 hr.

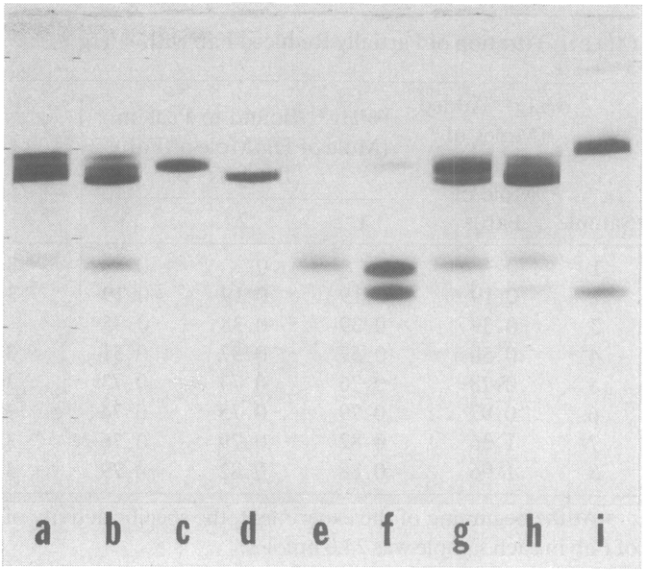


FIGURE 12: Identification of multiple components in Fab-Hg after electrophoresis in 10% polyacrylamide gels with 0.1% SDS–0.05 M sodium phosphate buffer (pH 7.0); (a and b) Fab-Hg; (c) Fab; (d) mercury derivative of light chain; (e) mercury derivative of Fd; (f) Fab-RA; (g) Fab-Hg mixed with Fab; (h) Fab-Hg mixed with mercury derivative of light chain; (i) IgG-RA. Pretreatment with SDS and heat was omitted in samples a–e, g, and h. These samples were in 0.1 M sodium acetate (pH 4.0) and were added (with sucrose) directly to the gels. Samples a and b were duplicates except that sample b was the first sample added to the series of gel tubes and sample a was the 15th and last sample. Sample f was in 0.1 M Tris-chloride (pH 8.0) and was made 1% in SDS and heated to 60° for 1 hr; sample i was in 5 mM Tris-chloride and was also made 1% in SDS and heated to 60° for 1 hr.

mercury derivative of Fab contained a group of two or three closely spaced bands that migrated in the approximate position of native Fab. In addition there was a faster component that varied in intensity in different runs and that corresponded in position to Fd. Above this component there was a faint smear of protein extending to the group of slower moving bands. A very faint component corresponding to light chain was also usually visible. There was some variation in the strength of these several bands from run to run. In addition, it was noted that there was an inverse correlation between the intensity of the uppermost band and that of the band corresponding to Fd (compare Figure 11c,d). When these gels were divided into 1-mm slices and counted, ^{203}Hg was found, in approximate proportion to staining intensity, to be associated with the triplet of bands in the upper region (these were not resolved clearly in the 1-mm slices) and with free Fd.

The variability in the strength of these bands suggested that the two subunits in Fab-Hg were participating in exchange reactions in SDS. The upper component in the triplet of bands seen in the gels was probably a dimer of Fd, the lower component a dimer of light chain, and the middle component a dimer of light chain and Fd, or native Fab. The presumptive Fd dimer tended to dissociate into the monomer during the course of a run, giving rise to the band in the position of free Fd and to the smear above it. An exchange reaction may also have occurred in acetic acid but the various components were not clearly resolved by gel filtration. It seemed probable that such exchange reactions would be favored by dissociating conditions and that they might not occur to an appreciable extent in "benign" solvents. In this case, in solvents such as acetate buffer (pH 4.0), Fab-Hg would not be a mixture of components but a homogeneous entity.

To obtain further evidence regarding the nature of Fab-Hg in both dissociating and nondissociating solvents it was necessary to: (1) confirm the nature of the triplet of bands found after electrophoresis of Fab-Hg in SDS–polyacrylamide gels and (2) determine whether or not these components are present in 0.1 M sodium acetate buffer (pH 4.0). Accordingly, mercury derivatives of free light chains and of Fd were prepared. Native Fab was reduced by incubation at 37° for 2 hr in 5 mM dithiothreitol, 0.1 M Tris-chloride (pH 8.0). The reduced protein was added to a column of Sephadex G-75 equilibrated at 4° with 1 M acetic acid made 0.1% in mercaptoethanol. Two peaks of approximately equal area were eluted as well as a small amount of aggregated material. The material in the first major peak was shown by electrophoresis in SDS–polyacrylamide gels to be Fd and the material in the second peak was light chain. An 0.8-ml aliquot of the fractions of maximum concentration (about 1 mg/ml) in each of these peaks was passed rapidly under N_2 pressure through a 0.8 × 15 cm column of Sephadex G-25 equilibrated with 0.1 M sodium acetate buffer (pH 4.0). The eluate fraction containing the reduced protein was collected with stirring into an approximately twofold molar excess of $^{203}Hg^{2+}$ also in acetate buffer. Each sample was then dialyzed against the same buffer with several changes of dialysis bag. After the fourth dialysis the concentration of ligand in these samples was: 0.4 mole of ^{203}Hg/mole of light-chain monomer (assumptions: $E^{1cm}_{1\%}$ at 278 nm = 15, mol wt 23,000) and 0.6 mole of ^{203}Hg/mole of Fd monomer (assumptions: $E^{1cm}_{1\%}$ at 278 nm = 15, mol wt 24,000).

The mercury derivatives of light chain and of Fd, as well as the mercury derivative of Fab, were analyzed by electrophoresis in polyacrylamide gels containing SDS with the results shown in Figure 12. Samples a and b were both from

TABLE III: Stability of Mercury in Fc-Hg and Fab-Hg.

Sample	Reagent[a]	Relative Content of $^{203}Hg^{2+}$ (%)[b] Fc-Hg	Fab-Hg
1	0.1 M sodium acetate, pH 4.0	100	100
2	0.1 M Tris-chloride, pH 8.0	99	94
3	0.001 M EDTA, pH 8.0	98	92
4	0.02 M EDTA, pH 8.0	97	89
5	0.001 M NaN_3, pH 8.0	100	97
6	0.02 M NaN_3, pH 8.0	99	91
7	0.001 M KCN, pH 8.0	97	90
8	0.02 M KCN, pH 8.0	88	63
9	0.001 M iodoacetamide, pH 8.0	86	91
10	0.02 M iodoacetamide, pH 8.0	42	25
11	0.001 M cysteine, pH 8.0	9	3
12	0.01 M cysteine, pH 8.0	3	
13	0.0005 M cystine, pH 8.0	95	87
14	1% SDS, pH 8.0	97	61
15	0.005 M $HgCl_2$, pH 4.0		0.8
16	0.01 M $HgCl_2$, pH 4.0	0.9	
17	0.005 M $AgNO_3$, pH 4.0		85
18	0.01 M $AgNO_3$, pH 4.0	89	

[a] Specific reaction conditions were as follows. Samples 1, 2, 3, 5, 7, 9, 11, 12, 13, and 14 (concentration of protein 0.6 mg/ml) were dialyzed for 36 hr at 4° (except 14–37°) against a 125-fold excess (v/v) of each reagent. Samples 4, 6, 8, and 10 (0.5 mg of protein) were incubated in 0.8 ml of reagent for 13 hr at 37° and then dialyzed for 24 hr at 4° against a 125-fold excess (v/v) of 0.1 M Tris-chloride (pH 8.0). Samples 15–18 (0.2 mg of protein) were incubated in 0.6 ml of reagent for 1 hr at room temperature and then dialyzed for 24 hr at 4° against a 400-fold excess (v/v) of 0.1 M sodium acetate buffer (pH 4.0). [b] The content of mercuric ion in each sample is expressed relative to that of the control (sample 1). The experimental error in these determinations is estimated to be approximately ±3%. The blank spaces mean no sample.

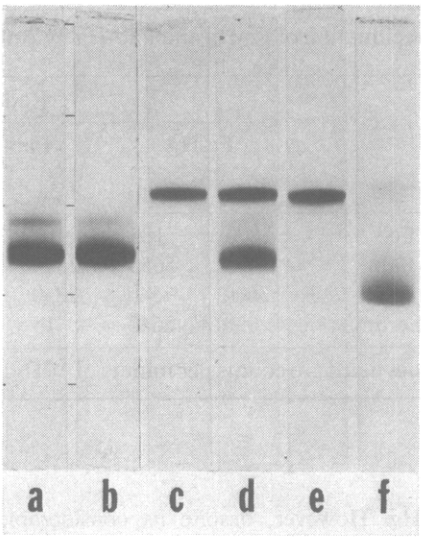


FIGURE 13: Absence of multiple components in Fab-Hg after electrophoresis in 7.5% polyacrylamide gels–0.1 M sodium acetate (pH 4.0). (a) Fab; (b) Fab-Hg; (c) mercury derivative of light chain; (d) Fab-Hg mixed with mercury derivative of light chain; (e) light-chain fraction obtained by gel filtration of Fab-RA in 1 M acetic acid; (f) Fd fraction obtained by gel filtration of Fab-RA in 1 M acetic acid. Samples a–d were in 0.1 M sodium acetate (pH 4.0). Samples e and f were in 1 M acetic acid.

the same preparation of Fab-Hg. However, sample a was the 15th and last sample to be applied to the gel tubes in this experiment whereas sample b was the first sample and therefore was exposed to 0.1% SDS for an additional 30 min before the electrophoretic run was started. The two samples yielded similar patterns except that there was less material in the uppermost band in sample b and correspondingly more material in a lower band. Native Fab (Figure 12c) migrated in the same position as the middle component of the group of three closely spaced bands in Fab-Hg. This was confirmed by mixing native Fab with Fab-Hg (Figure 12g) and observing the increase in stain corresponding to the middle component. The mercury derivative of light chain (Figure 12d) corresponded in mobility to the lowest component in the triplet of closely spaced bands and this was confirmed by mixing this sample with Fab-Hg (Figure 12h). The mercury derivative of Fd (Figure 12e) migrated in the same position as free Fd and this band corresponded also to a band seen in Fab-Hg. Since the strength of this latter component varied inversely with that of the uppermost band in Fab-Hg, the upper band is probably a dimer of Fd. Evidently, the mercury derivative of Fd may not dimerize and even if it does form a dimer, this is relatively unstable and dissociates readily during the electrophoretic run.

Thus, the major components found after electrophoresis of Fab-Hg in SDS gels corresponded in mobility to the monomer or dimer of Fd, to native Fab, and to the dimer of light chain. It remained to show that these components are not *all* present when Fab-Hg is analyzed in a nondissociating solvent. Accordingly, the mercury derivative of Fab and of light chain, as well as free light chain and Fd, were subjected to electrophoresis in polyacrylamide gels in 0.1 M sodium acetate buffer (pH 4.0). The results, shown in Figure 13, demonstrated that the mobility of Fab-Hg was the same as that of native Fab (Figure 13a,b). The minor component in these preparations is an aggregate of Fab. The mercury derivative of light chain (Figure 13c) was clearly resolved from Fab-Hg. Free light chain (Figure 13e) migrated in the same position as its mercury derivative and evidently dimerized in this solvent. Fd (Figure 13f) was also resolved from Fab-Hg.

These results imply that the variety of components found after electrophoresis of Fab-Hg in SDS is the result of a subunit exchange that occurs in detergent but not in "benign" solvents such as acetate buffer at pH 4.0. It could be concluded therefore that the structure of the mercury derivative of Fab in nondissociating solvents resembles closely that of native Fab with a single atom of mercury now associated with the two cysteine residues that were formerly united in the interchain disulfide bridge.

Stability of Mercury in Fc-Hg and Fab-Hg. The strength of binding of Hg^{2+} in these proteins was evaluated by treating the derivatives with a variety of reagents having affinity either for heavy metals or for sulfhydryl groups. Loss of ^{203}Hg was measured as a change in the specific radioactivity of the derivatives. The results of these experiments are summarized in Table III. EDTA, azide, and cystine were relatively ineffective in removing mercuric ion from either derivative. KCN and iodoacetamide at a concentration of 0.02 M were somewhat more effective in bringing about a loss of Hg^{2+}, especially

TABLE IV: Precipitation of Fc-Hg and Fab-Hg by Antisera.

	Radioactivity in Immune Precipitates and Supernatants[a]											
	Fc-Hg		Fab-Hg		Fc + Hg^{2+}		Fab + Hg^{2+}		Fc + Fab-Hg		Fab + Fc-Hg	
Serum	Ppt	Sup	Ppt	Sup	Ptp	Sup	Ppt	Sup	Ppt	Sup	Ppt	Sup
Anti-Gil Fc	5800	185	10	2420	700	7360			90	2380		
Anti-Gil Fab	25	5650	2510	110			190	2280			170	5180
Anti-Gil IgG1	5660	340	2210	405								
Normal Serum	30	5640	15	2330								

[a] The values listed are counts per minute of ^{203}Hg.

from Fab-Hg. However, despite its considerable stability, the mercury–sulfur bond can be dissociated and exposure to either nonradioactive Hg^{2+} or to a free sulfhydryl reagent, cysteine, resulted in the rapid and virtually complete displacement of the ^{203}Hg. Ag^{+} was much less effective than Hg^{2+} in accordance with its lower affinity for sulfhydryl groups (Gurd and Wilcox, 1956). In another experiment, all of the $^{203}Hg^{2+}$ was removed from Fc-Hg by treatment with a large excess of CNBr in 70% formic acid.

To determine whether the $^{203}Hg^{2+}$ in the derivatives might become complexed to disulfide bonds or other reactive groups in native immunoglobulins, a series of experiments was carried out in which Fc-Hg or Fab-Hg was incubated for 20 hr at 4° with a tenfold excess of nonradioactive protein (*e.g.*, Gil IgG, Fc, or Fab) in either 0.1 M sodium acetate buffer (pH 4.0) or 0.05 M sodium phosphate buffer (pH 7.0). The mixture of proteins was then resolved by gel filtration in acetate buffer (pH 4.0). In no case was there any migration of radioactively labeled Hg^{2+} from either derivative to any of the unlabeled proteins. If, however, the incubation and gel filtration were carried out in 1 M acetic acid, some $^{203}Hg^{2+}$ was transferred from the derivative to the previously unlabeled protein. The sites for binding mercuric ion in the recipient protein were not defined. However, it was shown that the interchain disulfide bridges were not involved since the transfer was not diminished after the reduction and alkylation of these bonds. The degree of transfer of Hg^{2+} varied according to the nature and concentration of the unlabeled protein, but was always greater from Fab-Hg than from Fc-Hg, consistent with the lability of the mercury–sulfur bond in Fab that had been noted previously in dissociating solvents.

These experiments demonstrate that although the binding of mercuric ion in these proteins is potentially reversible, the mercury–sulfur bond has a considerable degree of stability, provided that the derivative is kept in nondissociating solvents. The mercury derivative of Fab was somewhat less stable than the derivative of Fc.

Precipitability of Fc-Hg and Fab-Hg by Antisera. Analysis by immunodiffusion (*e.g.*, Figure 4) indicated that the mercury derivatives of Fc and Fab were each antigenically identical with their respective native proteins. Evidently, the rearrangement in disulfide bonding in Fc did not substantially affect its immunologic reactivity. An alternate possibility was that the reaction with antiserum released the bound Hg^{2+} with re-formation of the native molecule. Accordingly, the mercury-containing derivatives were now tested for the retention of bound mercury after precipitation by various antisera. An aliquot of each derivative (0.1 ml of a solution 0.7 mg/ml) was mixed with 1.0 ml of antiserum and incubated at 37° for 1 hr and at 4° for 36 hr. To test for nonspecific precipitation of unbound mercuric ion, free $^{203}Hg^{2+}$ was mixed with native protein and then reacted with antiserum. As a second control, Fab-Hg (Fc-Hg) was mixed with native Fc (Fab) and reacted with anti-Fc (anti-Fab). The samples were centrifuged and the radioactivity of the unwashed precipitates and supernatant solutions was determined (see Table IV). Fc-Hg was precipitated both by an antiserum prepared against the Gil Fc fragment and by one prepared against the whole Gil myeloma protein, but not by anti-Gil Fab. Conversely, Fab-Hg was precipitated by antisera prepared against Fab and IgG but not by anti-Gil Fc. There was no appreciable nonspecific precipitation of free $^{203}Hg^{2+}$ or of either derivative (*e.g.*, Fab-Hg) in the presence of the other immune system (Fc and anti-Fc). These results demonstrate not only that the mercury derivatives of Fc and Fab are antigenically related to the native proteins but that the bound $^{203}Hg^{2+}$ is retained in the immune precipitates. The data also confirm the purity and specificity of these proteins and antisera.

Discussion

We have described the preparation and properties of specific mercury derivatives of the Fab and Fc fragments of a human myeloma protein of subclass IgG1. These derivatives are prepared by selective reduction of the interchain disulfide bridges followed by reaction of the partially reduced protein with mercuric ion. A single atom of mercury becomes associated in a stable complex with each pair of reduced cysteine residues. In the presence of excess Hg^{2+}, more metal ions may be bound to the protein but these additional ions are easily removed by dialysis or gel filtration. Thus, the derivative of Fab contains approximately 1 and that of Fc approximately 2 moles of mercuric ion per mole of protein.

The characteristic pattern of interchain disulfide bonding in the Fab and Fc fragments leads to differences in the subunit structure of the mercury derivatives. In Fab a single disulfide bridge connects the light chain to Fd. The mercury derivative of Fab was indistinguishable from the native protein by several criteria. The two proteins migrated identically in polyacrylamide gel electrophoresis and in gel filtration in acetate buffer (pH 4.0). They gave a reaction of identity in double diffusion in agar with antiserum prepared against native Fab. Moreover, the derivative with its bound mercuric ion was quantitatively precipitated by the same antiserum. Presumably, Fab-Hg is identical with native Fab except that the interchain disulfide bond has been replaced by a bridge of the

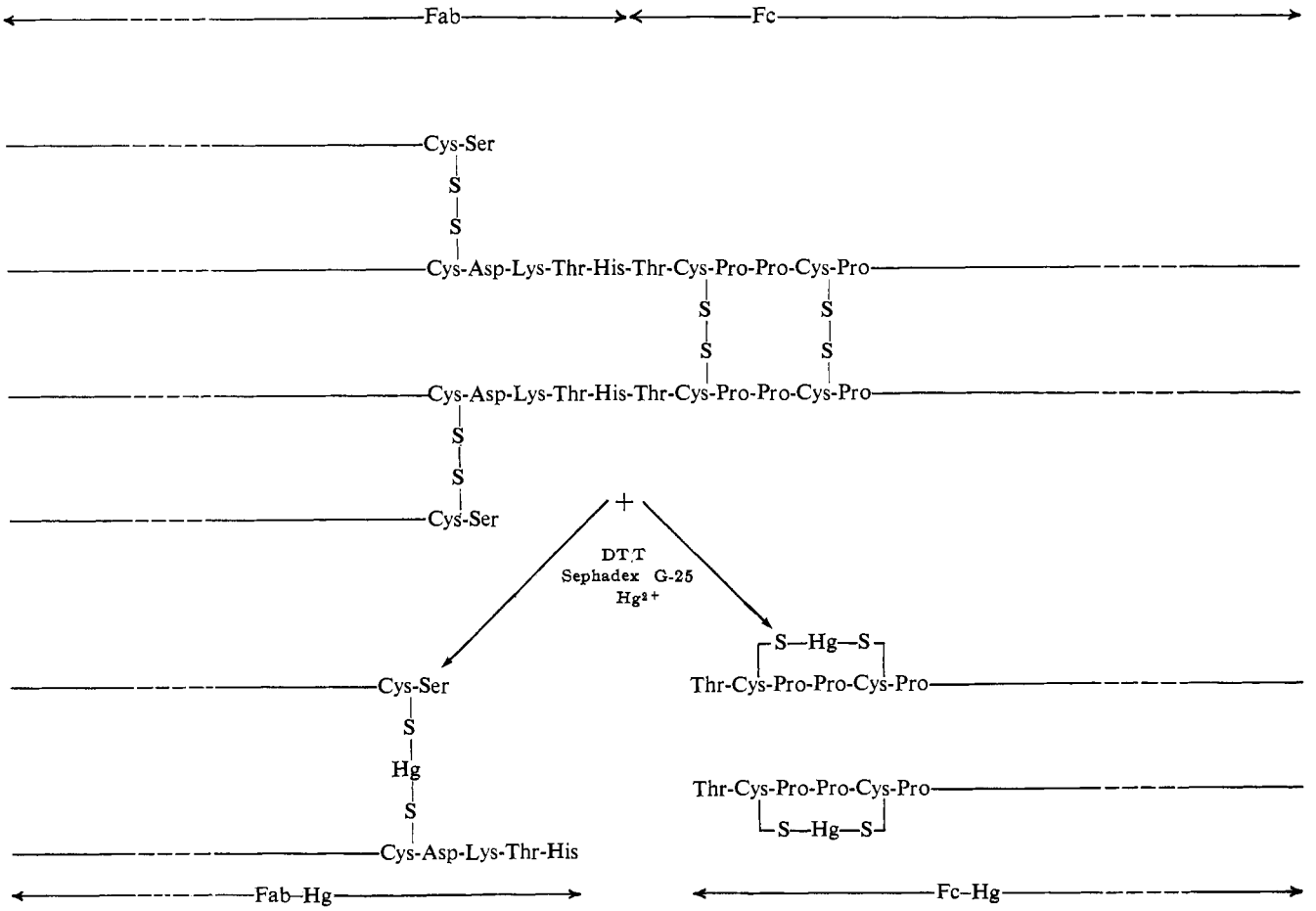


FIGURE 14: Proposed structures for mercury derivatives of Gil Fab and Fc. The position of cleavage by papain to form the Fab and Fc fragments in IgG1 was established by Steiner and Porter (1967) and by Press and Hogg (1970).

type S–Hg–S (Figure 14). However, the mercury–sulfur bond is less stable than the disulfide bond. Accordingly, when Fab-Hg is exposed to solvents such as SDS or acetic acid, the light chain and Fd piece dissociate and then reassemble in a variable manner to form mercury-containing dimers of light chain and Fd in addition to the mixed dimer, native Fab.

In contrast, the Fc fragment consists of a pair of identical subunits cross-linked by two disulfide bonds. These interchain bridges are very closely spaced, being separated by only two residues of proline (Figure 14). In the mercury derivative, the interchain disulfide bonds have presumably been replaced by bridges of the type S–Hg–S. Fc-Hg and native Fc were indistinguishable in polyacrylamide gel electrophoresis and gel filtration in acetate buffer (pH 4.0). The two proteins were antigenically identical in double diffusion with an antiserum to native Fc. The derivative with its retained mercury was completely precipitated by the same antiserum. However, electrophoresis in SDS–polyacrylamide gels and gel filtration in acetic acid demonstrated that Fc-Hg is a monomer. In these dissociating solvents the mercury derivative behaved identically with a preparation of Fc that had been separated into monomeric subunits by reduction and alkylation of the interchain disulfide bridges. These results indicate that at least in dissociating solvents Fc-Hg is not cross-linked by bonds of the type S–Hg–S, but that the former interchain bonds rearrange in the presence of Hg^{2+} to form intrachain bonds. A possible conformation for Fc-Hg became apparent upon construction of a space-filling molecular model of the region near the two interchain disulfide bridges. The separation of the two residues of half-cystine in each chain is such that an atom of mercury can be accommodated between them to form an intrachain loop of the type shown in Figure 14. This arrangement is possible with the peptide bond between the two proline residues in either the trans or the cis configuration. (That the peptide bond between adjacent proline residues may on occasion be in the cis form has been demonstrated in the X-ray diffraction studies of ribonuclease (Wyckoff *et al.*, 1967).)

It is difficult to establish unambiguously whether the Hg^{2+} in Fc-Hg is bound in an intrachain loop also in nondissociating solvents such as acetate buffer (pH 4.0). Since covalent interchain bridges are not required for maintaining the dimeric structure of Fc in these solvents, the state of the mercury–sulfur bonds (interchain *vs.* intrachain) could not be evaluated directly. However, studies with a smaller molecule that resembles Fc in its interchain bonding were helpful on this point. A peptide dimer composed of the 28 N-terminal residues of Fc, still cross-linked by the two interchain disulfide bridges, was obtained from a CNBr digest of Fc. The critical difference between this peptide and the native Fc fragment is that after reduction of the interchain disulfide bonds, the two chains in the peptide can be separated in solvents that do not dissociate the subunits of Fc. The peptide was reduced and reacted with mercuric ion under the same conditions used in the preparation of Fc-Hg. The subunit structure of the mercury derivative of the peptide was then examined by gel

filtration in acetate buffer (pH 4.0), a nondissociating solvent for Fc. The derivative of the peptide was a monomer in this solvent. This is consistent with the possibility that the mercury in this peptide derivative is held in an intrachain loop of the type shown for Fc-Hg in Figure 14. Extrapolation of these results to the Fc molecule may not be entirely valid since at pH 4.0, reduced and alkylated Fc, unlike the peptide, is held together by noncovalent forces. These interactions might well affect the mode of its reactions with mercury. Nonetheless, the results with the model compound demonstrate that it is possible to form the presumed loop structure in a nondissociating solvent. Therefore, the Hg^{2+} in Fc-Hg may also be bound in this configuration in such solvents.

An alternative approach for investigating the arrangement of the mercury–sulfur bonds was considered: the preparation of the mercury-containing peptide from the derivative of Fc. However, *even if* the S–Hg–S bonds were interchain in Fc-Hg, they would almost certainly rearrange to the favored intrachain form as soon as the peptide was released from the rest of the molecule and no longer subject to the noncovalent interactions that tend to preserve the dimeric structure of Fc. Accordingly the isolation of such a mercury-containing peptide from Fc-Hg has not been attempted.

The strength of binding of Hg^{2+} in these derivatives was investigated. Under most conditions the ligand was bound very firmly. Dialysis against a variety of buffers in the pH range 3–8 resulted in almost no loss of mercuric ion. When either derivative was mixed with a native immunoglobulin in acetate buffer (pH 4.0) or in phosphate buffer (pH 7.0), transfer of Hg^{2+} to the native protein did not occur. Moreover exposure of the derivatives to large excesses of reagents that might displace the ligand either by complexing with it or by binding to sulfhydryl groups led to relatively little loss of mercuric ion (Table III). For example, only when the concentration of iodoacetamide or KCN was increased to greater than a 1000-fold excess with respect to mercury was there a significant loss of $^{203}Hg^{2+}$. Nevertheless, the bonding between mercury and sulfur is reversible. Reaction with sulfhydryl compounds or with nonradioactive mercuric ion effectively displaced all the $^{203}Hg^{2+}$ from both derivatives.

There was a small but consistent difference between the two proteins in that mercuric ion could be displaced somewhat more easily from the derivative of Fab than from that of Fc. For example, after prolonged dialysis in 1% SDS, Fab-Hg lost about 40% of the bound Hg^{2+} whereas Fc-Hg lost almost none. The increased lability of the mercury–sulfur bond in the Fab derivative was also demonstrated by its propensity to undergo subunit exchange in SDS. It seems, therefore, that the ring structure in Fc-Hg provides additional stability for the binding of mercuric ion.

The binding of mercuric ion to other proteins has been the subject of a number of investigations. Although several amino acid residues are capable of reacting with Hg^{2+}, the affinity of this ligand for sulfhydryl groups is far greater than for the other functional groups on proteins. The interactions of Hg^{2+} with simple mercaptans such as cysteine and glutathione were studied by Stricks and Kolthoff (1953). It was determined that in these compounds the equilibrium constants for the reaction

$$Hg^{2+} + 2RS^- \rightleftharpoons RS\text{–}Hg\text{–}SR$$

were on the order of 10^{41}–10^{45}. The affinity of Hg^{2+} for protein sulfhydryl groups may be considerably modified but is usually still very great.

The possibility of preparing a crystallizable dimer of serum albumin by cross-linking with mercury those molecules that have a reactive sulfhydryl group was demonstrated by Hughes (1947). The kinetics of formation and dissociation of such albumin dimers joined by Hg^{2+} as well as by larger bifunctional organic mercurials were subsequently investigated in detail (Edelhoch *et al.*, 1953; Edsall *et al.*, 1954). More recently, mercuric ions have been introduced into the disulfide bonds of a number of enzymes. This has been accomplished by reduction followed by reaction with Hg^{2+}. With ribonuclease, the transformation of the disulfide bond connecting half-cystine residues IV and V into a bridge of the type S–Hg–S was accompanied by relatively little change in the structure or function of the protein (Sperling *et al.*, 1969). Even when all four disulfide bonds were reacted with Hg^{2+} the product retained some of its enzymatic activity (Sperling and Steinberg, 1971). In the case of papain, a derivative has been prepared with one ligand of mercury attached to the sulfhydryl group in the catalytic site and a second ligand interposed within a specific disulfide bond (Arnon and Shapira, 1969). The modified enzyme was slightly altered in its kinetic properties but its immunological reactivity was identical with that of native papain. Dialysis of the derivatives of ribonuclease and papain against EDTA resulted in loss of mercury (Arnon and Shapira, 1969; Sperling *et al.*, 1969). Moreover, precipitation of the monomercury derivative of ribonuclease with antisera removed the ligand from the protein (Sperling *et al.*, 1969). Since in our studies mercury was not removed from Fab-Hg and Fc-Hg by comparable treatment, it would seem that the ligand is bound more strongly in the derivatives of the immunoglobulins than in the derivatives of these two enzymes.

The introduction of Hg^{2+} into specific protein disulfide bonds may provide a useful method for preparing heavy metal derivatives for X-ray crystallographic studies. Crystals of the derivative of ribonuclease with mercuric ion inserted into cystine bond IV–V resembled crystals of native ribonuclease in space group and in unit cell dimensions (Sperling *et al.*, 1969). Detailed X-ray diffraction studies with these crystals have not yet been reported. In our studies, the crystal structure of the mercury derivative of Fc was closely related to, but not isomorphous with, that of the native protein. This could be due to a change in molecular packing resulting from a rearrangement in the disulfide bonding. It is also possible that modifying the conditions of crystallization or carrying out the reduction and mercury reaction on crystals of Fc itself might yield an isomorphous product.

In Fab the single disulfide bridge connecting the light chain to Fd was apparently transformed by reaction with mercury into a bridge of the type S–Hg–S and continued to cross-link the two subunits. Although we have so far not been able to prepare crystals of Gil Fab, crystallizable Fab and Fab′ fragments have been obtained from several human myeloma proteins (Rossi and Nisonoff, 1968; Rossi *et al.*, 1969). In addition, two intact IgG1 proteins have recently been crystallized (Terry *et al.*, 1968; Edmundson *et al.*, 1970). X-Ray diffraction studies of some of these proteins are in progress (Avey *et al.*, 1968; Humphrey *et al.*, 1969; Edmundson *et al.*, 1970; Sarma *et al.*, 1971). It will be of considerable interest to determine whether the addition of mercury to the interchain disulfide bridges in these proteins will yield useful derivatives for the structure work.

Rabbit immunoglobulin provides an interesting contrast to the human protein in that the predominant type of rabbit IgG has only a single disulfide bond between its two heavy chains (Palmer and Nisonoff, 1964). In the usual preparation

of Fc fragment from the rabbit protein (Porter, 1959) the portion of the molecule containing this disulfide bond may be lost as a result of digestion with papain. However, by controlling the conditions of the enzymatic digestion it is possible to obtain a relatively good yield of Fc fragment with the interchain disulfide bond intact. This material is readily crystallizable and the preparation of a derivative with mercuric ion interposed within the disulfide bridge is currently in progress (R. Horner and L. Steiner, unpublished experiments). In analogy with the Gil Fab fragment, it is anticipated that the mercury derivative of the Fc fragment from rabbit IgG will retain its cross-linked structure; it may accordingly be useful for crystallographic studies.

A number of bifunctional reagents containing two reactive atoms of mercury have been described (see review by Blake, 1968), and these might also serve as useful cross-linking reagents. Such compounds would have the advantage of providing greater electron density for diffraction studies. The dimerization of serum albumin by a bivalent organic mercurial has been reported (Edsall *et al.*, 1954), but the cross-linking of cysteine residues in other proteins with such reagents remains to be explored.

Acknowledgments

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